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Holoprosencephaly in RSH/Smith-Lemli-Opitz Syndrome: Does Abnormal Cholesterol Metabolism Affect the Function of *Sonic Hedgehog?*

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The RSH/Smith-Lemli-Opitz syndrome (RSH/SLOS) is an autosomal recessive malformation syndrome associated with increased levels of 7-dehydrocholesterol (7-DHC) and a defect of cholesterol biosynthesis at the level of 3ß-hydroxy-steroid-Δ^7-reductase (7-DHC reductase). Because rats exposed to inhibitors of 7-DHC reductase during development have a high frequency of holoprosencephaly (HPE) [Roux et al., 1979], we have undertaken a search for biochemical evidence of RSH/SLOS and other possible defects of sterol metabolism among patients with various forms of HPE. We describe 4 patients, one with semilobar HPE and three others with less complete forms of the HPE sequence, in whom we have made a biochemical diagnosis of RSH/SLOS. The clinical and biochemical spectrum of these and other patients with RSH/SLOS suggests a role of abnormal sterol metabolism in the pathogenesis of their malformations. The association of HPE and RSH/SLOS is discussed in light of the recent discoveries that mutations in the embryonic patterning gene, *Sonic Hedgehog (SHH)*, can cause HPE in humans and that the sonic hedgehog protein product undergoes autoproteolysis to form a cholesterol-modified active product. These clinical, biochemical, and molecular studies suggest that HPE and other malformations in SLOS may be caused by incomplete or abnormal modification of the sonic hedgehog protein and, possibly, other patterning proteins of the hedgehog class, a hypothesis testable in somatic cell systems.

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INTRODUCTION

The RSH/Smith-Lemli-Opitz syndrome (RSH/SLOS) is a relatively common, autosomal recessive malformation syndrome comprising psychomotor and growth retardation, cleft palate, hypospadias, postaxial polydactyly, and distinct craniofacial abnormalities [Smith et al., 1964; Opitz et al., 1969; Curry et al., 1987]. In addition to the characteristic craniofacial defects of RSH/SLOS such as microcephaly, ptosis, inner epicanthal folds, posterior cleft palate, and micrognathia, a number of more serious craniofacial and central nervous system defects are seen occasionally in RSH/SLOS patients. These include partial or complete agenesis of the corpus callosum, cerebellar hypoplasia, anterior fusion of the cerebral hemispheres, and arhinencephaly [Curry et al., 1987; McKeever and Young, 1990]. Although some of these malformations are elements of the holoprosencephaly (HPE) sequence, more complete forms of HPE have not previously been associated with RSH/SLOS.

Recently, Tint et al. [1994] reported an apparent primary disorder of cholesterol biosynthesis in 5 patients with classical RSH/SLOS who had severe hypocholesterolemia and increased plasma levels of 7-dehydrocholesterol (7-DHC), the immediate precursor of cholesterol in the Kandutsch-Russell pathway for cholesterol biosynthesis. Since then, almost all patients with a firm clinical diagnosis of RSH/SLOS have been found to have the same qualitative abnormality of sterol metabolism [Tint et al., 1995;

Cunniff et al., 1997]. Moreover, evidence for a deficiency of 3ß-hydroxysteroid-Δ^7-reductase (7-DHC reductase), the enzyme that converts 7-DHC to cholesterol, in RSH/SLOS fibroblasts has also been shown [Shefer et al., 1995]. These clinical and biochemical findings brought into focus a series of teratological studies [Roux and Aubry, 1966; Roux et al., 1980; Repetto et al., 1990] that demonstrated that Triparanol or AY-9944, both potent inhibitors of cholesterol biosynthesis, fed to pregnant rats produced a high frequency of fetal skeletal and craniofacial malformations, including pituitary agenesis and HPE. In addition, the treated rats had abnormal plasma sterol profiles indistinguishable from those of RSH/SLOS [Repetto et al., 1990].

This remarkable convergence of clinical and biochemical teratology has not only provided a means to determine the full clinical spectrum of RSH/SLOS through simple laboratory testing but also suggested other clinical syndromes to test for evidence of RSH/SLOS. Because of our long-standing interest in HPE, we began a systemic survey of available diagnostic material from patients with HPE to determine if more severe forms of HPE could occur in RSH/SLOS, which then escape diagnosis because of the lack of craniofacial clues important for the diagnosis of RSH/SLOS. We have identified and present here 4 patients with biochemically confirmed RSH/SLOS and malformations of the HPE sequence. We also discuss the significance of these findings in light of recent evidence from our and other laboratories linking cholesterol metabolism and HPE to the function of the vertebrate early embryonic patterning gene, *Sonic Hedgehog (SHH).*

MATERIALS AND METHODS

Plasma or tissue samples were obtained as 1) diagnostic specimens from patients with a known or suspected clinical diagnosis of HPE or SLOS, or 2) normal and pathological plasma samples from our diagnostic sample archives. Most plasma samples were collected without regard to the degree of fasting and were stored frozen at -20 °C or colder until use. The collection and use of the patient samples was approved by the Institutional Review Boards of the principal investigators (R.I.K., M.M.).

Plasma sterol concentrations were determined by gas chromatography/ion-ratio mass spectrometry [Kelley, 1995]. The method quantifies all saturated and mono-, di- and triene neutral sterols, of which cholesterol, 7-DHC, cholestanol (dihydrocholesterol), desmosterol (cholesta-5,24-dien-3ß-ol), and lathosterol (cholest-7-en-3ß-ol) are the most abundant in human plasma and cultured cells.

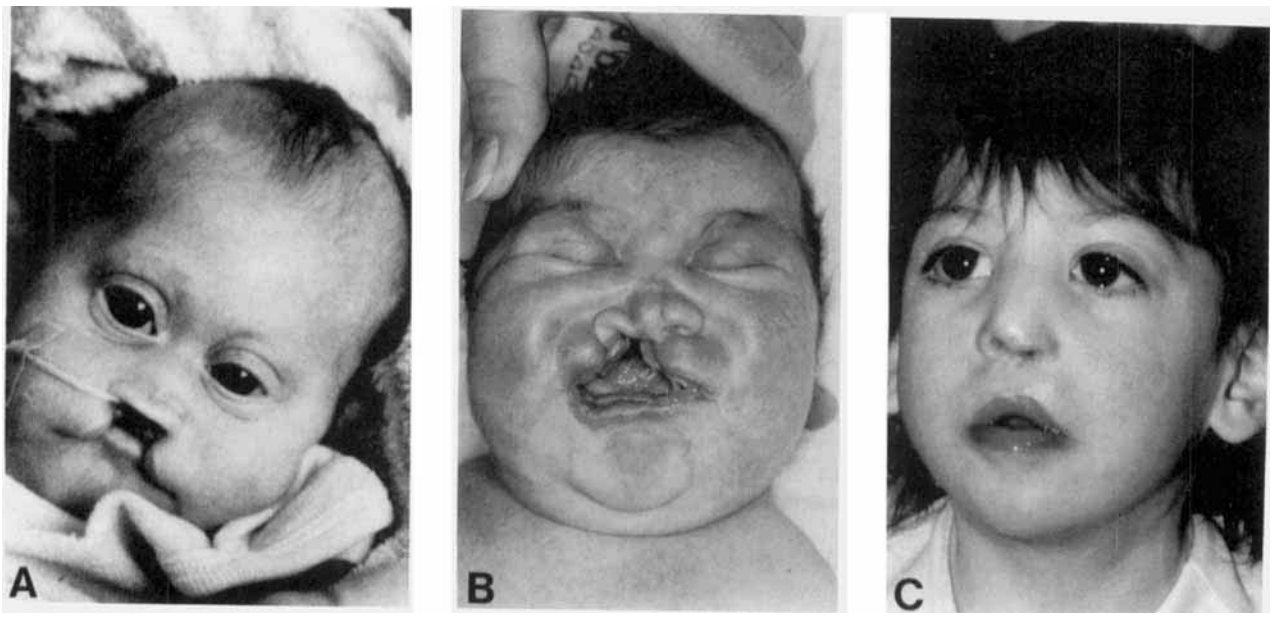
Lymphoblast or chorionic villus cultures were expanded in sealed flasks in bicarbonate-buffered RPMI 1640 (1 mM glucose) with 10% (v/v) Serumax (Sigma). In preparation for quantification of 7-DHC, cultures were split 1:2 weekly for two cycles into the same culture medium modified by replacing the 10% Serumax with the same amount of cholesterol-depleted fetal bovine serum [Gibson et al., 1990] supplemented with a growth factor mixture at the recommended concentration (Sigma #I-1884). The final cholesterol concentration of the culture medium was less than 1 µg/ml. After reaching saturation density, the cells were harvested and analyzed as described elsewhere for cultured skin fibroblasts [Kelley, 1995]. All normal ranges are given as mean of N samples ± SD.

CASE REPORTS

Patient 1, previously reported in abstract [Muenke et al., 1994b], was born at term to nonconsanguineous parents following a pregnancy complicated by oligohydramnios in the last trimester. At birth, the baby was found to have severe craniofacial abnormalities including hypotelorism, proptosis, a single nostril nose, absent premaxilla, posterior cleft palate, and redundant nuchal skin (Fig. 1A). A cranial CT examination demonstrated semilobar HPE. Extracranial anomalies included postminimus polydactyly of the right hand and mild cutaneous syndactyly of toes 2 and 3. The external genitalia appeared to be female with a mildly enlarged clitoris, but the peripheral blood karyotype was 46,XY. The child was weak and hypotonic and died shortly after birth. Although the diagnosis of RSH/SLOS was not suspected at the time of death, decreased cholesterol synthesis and a markedly increased 7-DHC/cholesterol ratio of 0.507 (nl 0.0015 ± 0.0005, N=16) were later demonstrated in cultured lymphoblasts.

Patient 2 was a term, 2.85 kg infant who had been found by routine mid-gestation ultrasound examination to have a cleft lip and palate. Amniocentesis was performed and a normal (46,XY) karyotype found. At delivery, the infant was severely hypotonic, appeared growth-retarded, and had microcephaly, a unilateral cleft lip with absent premaxilla, cleft palate, micrognathia, microglossia, inner epicanthal folds, prominent infraorbital creases, excessive posterior nuchal skin, all whorl patterns on fingertips, and female external genitalia (Fig. 1B). The baby developed progressive respiratory distress and died at age 3 days. At autopsy, there was decreased pulmonary lobation, a primum atrial septal defect, abdominal testes, and duplicate blind-pouch vaginas. There were no gross structural abnormalities of the brain other than its small size (230 g vs. 359 g expected for gestational age of 38 w). A clinical diagnosis of RSH/SLOS was made and confirmed by plasma sterol analysis, which showed a cholesterol level of 8.7 mg/dl (normal, 65.9 ± 8.1; N=14), and 7-DHC level of 9.4 mg/dl (normal, 0.010 ± 0.005; N=68).

Patient 3 was a 3-year-old girl with moderate mental retardation and failure-to-thrive. She was born at term to nonconsanguineous parents and was



found to have microcephaly, bilateral ptosis, epicanthal folds, midline cleft lip and palate, bilateral clubfeet, and syndactyly of toes 2 and 3 (Fig. 1C). The cleft lip and palate were repaired at 6 months and 2 years, respectively. Both central upper incisors were also found to be absent. At 3 years, her length, weight, and head circumference were below the 3rd centile. Although she had severe developmental delay, she was able to communicate using several signs. Because of the malformations and facial characteristics of RSH/SLOS, a plasma sample was submitted for sterol analysis and found to have a diagnostically increased level of 7-DHC of 0.5 mg/dl (normal, 0.010 ± 0.005; N=68) but, surprisingly, a normal level of cholesterol of 179 mg/dl.

Patient 4 was a severely growth-retarded fetus born at 34 weeks gestation to nonconsanguineous parents following several months monitoring for growth retardation and severe oligohydramnios. Culture of a transabdominal CVS tissue showed a normal 46,XY karyotype. The parents' previous child was stillborn at 35 weeks gestation and was found at delivery to have severe growth retardation, midline cleft lip, postaxial polydactyly and 2-3 toe syndactyly of both feet, a complex cardiac defect, and female genitalia (karyotype not done). At delivery, patient 4 was stillborn and had similar malformations including notching of the upper lip, midline cleft of the soft palate, rhizomelic limb shortness, postaxial polydactyly of the right hand and both feet, and bilateral syndactyly of toes 2, 3 and 4. The genitalia appeared female. Bilateral renal agenesis was found at autopsy. Because of autolysis, no description of the brain was possible. A diagnosis of type II RSH/SLOS was made for both sibs. Although blood could not be obtained from patient 4 for sterol analysis, cultured CVS cells were submitted for sterol analysis and found to have a markedly increased 7-DHC/cholesterol ratio of 0.635 (nl 0.0007 ± 0.0002, N=6), consistent with the diagnosis of RSH/SLOS.

RESULTS

Of the first 50 lymphoblast lines examined from patients with autosomal recessive or sporadic HPE, only one (patient 1) was found to have a markedly increased 7-DHC/cholesterol ratio and a diene sterol pattern indistinguishable from that of patients with classic RSH/SLOS. In addition, both parents of patient 1 had levels of 7DHC that were more than 3 SD above normal, similar to the 7-DHC/cholesterol ratio of lymphoblasts from obligate heterozygotes for RSH/SLOS (data not shown). The cultured lymphoblasts of the mother of another infant with apparently autosomal recessive HPE was also found to have a 7-DHC/cholesterol ratio in the RSH/SLOS heterozygote range, but testable material from the propositus was not available. None of the other HPE patients for whom detailed descriptions were available had other cranial or extracranial malformations strongly suggestive of RSH/SLOS. Furthermore, no

sterol abnormalities were detected in 75 plasma samples collected prospectively from patients with all forms of HPE.

Phenotype analysis of the 4 patients in this series showed that, apart from the malformations that are elements of the HPE sequence, the range of external and internal malformations in the patients were typical of RSH/SLOS. All 4 had syndactyly of toes 2 and 3, which occurs in more than 95% of RSH/SLOS patients [Cunniff et al., 1997], and other typical RSH/SLOS malformations, such as micrognathia (4 of 4), cleft palate (4 of 4), and ambiguous genitalia (3/4). The 4 patients in this report are included in our larger series [Cunniff et al., 1997] of 80 patients with biochemically confirmed RSH/SLOS, all of whom except patient 1 were ascertained by various clinical criteria other than HPE. Excluding patient 1, the series of 79 patients included 67 index cases and 12 affected relatives. Thus, some part of the HPE sequence occurred in approximately 4% of these clinically ascertained RSH/SLOS patients. However, because some malformations of the HPE sequence, such as single central incisor and incomplete separation of frontal lobes, would not be evident in young infants without radiological studies, and because fetuses with RSH/SLOS may die in utero because of the severity of their malformations, the incidence of the HPE sequence in a completely ascertained population of RSH/SLOS may be higher. We have also not included several patients with isolated agenesis of the corpus callosum among our RSH/SLOS cases with HPE, because agenesis of the corpus callosum can have various causes. Nevertheless, because we found only a single confirmed case of RSH/SLOS among the first 125 HPE patients (50 lymphoblasts and 75 plasma samples) screened for biochemical evidence of RSH/SLOS, RSH/SLOS is probably an uncommon cause of the more complete forms of the HPE sequence referred to our laboratory for molecular testing.

DISCUSSION

The discovery of abnormal cholesterol metabolism in RSH/SLOS has provided geneticists with a new biochemical approach to understanding abnormal morphogenesis in a syndrome with diverse malformations involving essentially all tissues. Although there are other metabolic malformation syndromes, such as Zellweger syndrome, RSH/SLOS is currently the best example of a syndrome in which a discrete block in a single metabolic pathway leads to such diverse effects on morphogenesis. One of the more important dividends of the discovery of abnormal levels of 7-DHC in RSH/SLOS is that the full clinical spectrum of RSH/SLOS can now be determined, allowing the diagnosis of RSH/SLOS to be assigned to or excluded from the more questionable or equivocal cases. Because some lethally affected RSH/SLOS infants and fetuses have few extracranial malformations, and because the facial malformations of HPE can obscure the diagnostically important facial characteristics of RSH/SLOS, the recognition that the HPE sequence can occur in RSH/SLOS is important.

HPE is a heterogeneous malformation sequence of the midface and forebrain [Muenke et al., 1994a]. There is a wide range of clinical severity of HPE, from the most severe *alobar* forms with cyclopia and absent septation of the cerebral hemispheres, to intermediate *semilobar* forms with partial septation of the cerebral hemispheres, to *microforms* comprising microcephaly, hypotelorism, midfacial clefting, a single central incisor, and other defects. There are both autosomal dominant and autosomal recessive monogenic HPE syndromes, often with a high degree of intrafamilial variability, from apparently isolated median cleft lip or single central incisor to alobar HPE. Based on our study of chromosomal deletions, we have defined 4 minimal critical regions for HPE, *HPE1* in 21q22.3 [Muenke et al., 1995], *HPE2* in 2p21 [Schell et al., 1996], *HPE3* in 7q36 [Gurrieri et al., 1993; Muenke et al., 1994a], and *HPE4* in 18p11.3 [Overhauser et al., 1995]. None of these chromosomal regions has been implicated in the cause of RSH/SLOS, for which 7q32.1 is the leading candidate chromosomal region [Alley et al., 1995]. Thus, the genetic or biochemical mechanism by which HPE occasionally arises in RSH/SLOS has been obscure.

The analysis of the sterol levels in our larger series of RSH/SLOS patients [Cunniff et al., 1997] has established that, whereas there is a poor correlation between the severity of RSH/SLOS and the plasma level of 7-DHC, there is a strong inverse correlation between the number of malformations and the level of plasma cholesterol at diagnosis. The apparent importance of the level of cholesterol in determining the severity of malformations in RSH/SLOS and its possible role in the development of HPE in RSH/SLOS was also suggested by the work of Barbu et al. [1984, 1988], who found that cholesterol fed to AY-9944-treated rats early in pregnancy could prevent HPE and other malformations in their fetuses despite the persistence of high levels of 7-DHC. Because cholesterol is not only the precursor of many other compounds, such as steroid hormones and bile acids, but also one of the major lipids of vertebrate cell and mitochondrial membranes, it is perhaps not surprising that a deficiency of a critical membrane lipid could lead to many different abnormalities of morphogenesis, which involves a multiplicity of cell-cell interactions.

Although there is much to be explained regarding the role of defective cholesterol biosynthesis in the abnormal morphogenesis of RSH/SLOS, the surprising discovery by Porter et al. [1996b] that the protein product of the prototypic embryonic segmentation gene, *hedgehog*, undergoes autoprocessing and covalent linkage with cholesterol suggests a novel mechanism for abnormal morphogenesis manifest as HPE in RSH/SLOS. *Hedgehog* in Drosophila and its homologous gene in vertebrates, *Sonic hedgehog*, are genes involved in the formation of embryonic segmental organizing centers [Nüsslein-Volhard and Wieschaus, 1980]. In

vertebrates, *Sonic Hedgehog* has been shown to have important patterning effects on tissues as diverse as the central nervous system, eyes, vertebral system and limbs [Echelard et al., 1993; Chang et al., 1994; Johnson et al., 1994; Ekker et al., 1995; Roelink et al., 1995]. As shown in a recent series of studies [Lee et al., 1994; Porter et al., 1995; Porter et al., 1996b], hedgehog proteins undergo autoproteolytic processing, forming a cholesterol-modified, ~19 kD amino-termimal segment (Hh-Np) that appears to embody all of the patterning activity of the Hh protein. The attachment of cholesterol to Hh-Np is apparently essential for the regional localization and concentration of Hh in various organizing centers of the developing Drosophila embryo [Porter et al., 1996a].

The apparently critical role of cholesterol in creating a functioning Hh-Np protein in Drosophila and of related signaling proteins in vertebrates suggests at least two mechanisms for the development of HPE and, possibly, limb and other malformations in RSH/SLOS. Because of the demonstrated inverse correlation of the severity of malformations with the plasma cholesterol level in RSH/SLOS patients, a deficiency of cholesterol may lead to incomplete processing of the Sonic Hedgehog protein, SHH, and deficient SHH-Np signaling, assuming that the concentration of cholesterol falls below a critical, but as yet unknown, catalytic level. Alternatively, because the lowest cholesterol levels in RSH/SLOS necessarily produce the highest 7-DHC/cholesterol ratios, simple competition between 7-DHC and cholesterol for covalent linkage to SHH-Np may lead to an increased proportion of 7-DHC-modified SHH-Np protein, which may possess no or only weak signaling. A direct association between *Sonic hedgehog* genes and HPE has also been made by the report of cyclopia in mice lacking *Sonic hedgehog* function [Chiang et al., 1996] and by our recent discovery of mutations of human *Sonic Hedgehog* in classical, autosomal dominant HPE [Roessler et al., 1996]. Although the association between the mutations of *Sonic Hedgehog* and HPE is firmly established in these families, the cause of the extreme variability of HPE expression in some HPE families is not clear.

Although probably very little cholesterol is transported from the mother to the human fetus through the placenta [Carr and Simpson, 1982; Bellknap and Dietschy, 1988], it is not unlikely that some maternal cholesterol diffuses to the developing embryo from the surrounding maternal tissues and fluids before the fetal membranes and placenta unit are fully developed. Because expression of Shh in the mesoderm underlying the anterior neural plate occurs very early in embryogenesis, such maternally derived cholesterol may influence the processing of Shh in the same way that cholesterol fed to AY-9944-treated pregnant rats prevents the development of HPE in the fetal rats. However, because the intrinsic rates of cholesterol synthesis in embryonic tissues during the first 8 weeks of gestation are unknown, the relative contribution of maternal cholesterol to cellular cholesterol in the developing forebrain and midface is also unknown.

The discovery of a role of cholesterol in the formation of active hedgehog proteins has suggested a molecular mechanism for HPE, and possibly malformations in other structures influenced by *Sonic Hedgehog* in RSH/SLOS. However, there remain many questions about the influence of both fetal and maternal cholesterol metabolism on normal embryonic and fetal development that must be answered before we can determine the role, if any, of *Sonic Hedgehog* in RSH/SLOS malformations. Similar questions regarding the role of fetal and maternal cholesterol metabolism in other genetic forms of HPE should also be asked. Although it is unlikely that cholesterol metabolism is directly involved in all forms of HPE, the fundamental importance of cholesterol in the processing and function of the Sonic Hedgehog protein, the association of mutations in *Sonic Hedgehog* with autosomal dominant HPE, and the wide intrinsic and nutritional variation in human blood cholesterol levels together suggest at least one possible mechanism underlying the highly variable expression of autosomal dominant HPE. Moreover, because Sonic Hedgehog has an important role in multiple embryonic tissues, and because there may be other human embryonic patterning proteins that undergo cholesterol modification, abnormal processing of Hedgehog proteins secondary to abnormal cholesterol metabolism may have a role in the development of other malformations of RSH/SLOS.

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